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Yersinia pestis Yop secretion protein F: Purification, characterization, and protective efficacy against bubonic plague

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Abstract

Yersinia pestis is a gram-negative human pathogen that uses a type III secretion system to deliver virulence factors into human hosts. The delivery is contact-dependent and it has been proposed that polymerization of Yop secretion protein F (YscF) is used to puncture mammalian cell membranes to facilitate delivery of *Yersinia* outer protein effectors into host cells. To evaluate the potential immunogenicity and protective efficacy of YscF against *Y. pestis*, we used a purified recombinant YscF protein as a potential vaccine candidate in a mouse subcutaneous infection model. YscF was expressed and purified from *Escherichia coli* by immobilized metal-ion affinity chromatography and protein identity was confirmed by ion trap mass spectrometry. The recombinant protein was highly α-helical and formed relatively stable aggregates under physiological conditions. The properties were consistent with behavior expected for the native YscF, suggesting that the antigen was properly folded. Ten mice were inoculated subcutaneously, administered booster injections after one month, and challenged with 130 LD_{50} of wild type *Y. pestis* CO92. Six animals in the vaccinated group but none in the control group survived the challenge. The vaccinated animals produced high levels of specific antibodies against YscF as determined by Western blot. The data were statistically significant ($P = 0.053$ by two-tailed Fisher's test), suggesting that the YscF protein can provide a protective immune response against lethal plague challenge during subcutaneous plague infection.
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Keywords: *Yersinia pestis*; Type III secretion; YscF; YscF; Vaccine

Yersinia pestis is a gram-negative human pathogen responsible for bubonic and pneumonic plague. Currently, a US licensed vaccine for use against plague in not available. However, a histidine-tagged recombinant fusion protein consisting of *Y. pestis* Fraction 1 capsular antigen (F1) and the V antigen (F1–V) has been shown to protect experimental mice against pneumonic as well as bubonic plague [1,2]. F1 is a 17 kDa capsule protein encoded by the *Y. pestis* pFra plasmid [3,4]. Numerous studies validate the efficacy of this highly immunogenic protein as a solitary protective antigen [5–7] against bubonic plague by encapsulated strains, but not against strains without capsule or against aerosol challenge [2,3]. The virulence (V) antigen is a 37 kDa secreted protein that is encoded by the 70-kb pCD1 plasmid [8,9]. As a multifunctional protein, the V antigen is involved with numerous aspects of plague pathogenicity [10–12]. As a solitary recombinant protein vaccine candidate, V antigen has been shown to provide significant protection against both encapsulated and non-encapsulated *Y. pestis* [2,13].

The recombinant F1–V fusion protein demonstrates promise as an effective vaccine against both bubonic and pneumonic plague. However, the nature of the two-component fusion protein could allow for the development

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14. ABSTRACT

Yersinia pestis is a gram-negative human pathogen that uses a type III secretion system to deliver virulence factors into human hosts. The delivery is contact-dependent and it has been proposed that polymerization of Yop secretion protein F (YscF) is used to puncture mammalian cell membranes to facilitate delivery of Yersinia outer protein effectors into host cells. To evaluate the potential immunogenicity and protective efficacy of YscF against Y. pestis, we used a purified recombinant YscF protein as a potential vaccine candidate in a mouse subcutaneous infection model. YscF was expressed and purified from Escherichia coli by immobilized metal-ion affinity chromatography and protein identity was confirmed by ion trap mass spectrometry. The recombinant protein was highly alpha-helical and formed relatively stable aggregates under physiological conditions. The properties were consistent with behavior expected for the native YscF, suggesting that the antigen was properly folded. Ten mice were inoculated subcutaneously, administered booster injections after one month, and challenged with 130 LD(50) of wild type Y. pestis CO92. Six animals in the vaccinated group but none in the control group survived the challenge. The vaccinated animals produced high levels of specific antibodies against YscF as determined by Western blot. The data were statistically significant (P=0.053 by two-tailed Fisher's test), suggesting that the YscF protein can provide a protective immune response against lethal plague challenge during subcutaneous plague infection.

15. SUBJECT TERMS
Yersina pestis, plague, type III secretion system, YscF, vaccine

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of genetically engineered strains of *Y. pestis* capable of bypassing F1–V vaccine induced immunity. Complex vaccines made of many constituents, including F1–V, may prove to be more difficult to counter. For this purpose, it is necessary to elucidate additional *Y. pestis* antigens with the capacity to induce a protective immune response against plague.

Yersinia pestis uses a contact-dependent type III secretion system to deliver virulence factors into host cells. The delivery system genes are located on the 70 kb pCD1 plasmid which codes for over 90 ORFs,[1] 44 of which are thought to code for TTSS proteins [14]. Twenty-nine proteins are thought to assemble into a macromolecular structure, referred to as the injectosome, that delivers at least six Yop effectors: YopH, YopE, YopM, YpkA, YopK, and YopJ, into target mammalian host cells. It is hypothesized that YopB, YopD, and LcrV form a pore in the mammalian cell membrane through which all of the other effectors are delivered.

The conduit connecting the bacterium and mammalian cell in the delivery process is known to have an opening with a diameter of approximately 2 nm [15]. This channel is considered to be large enough for the transport of partially unfolded proteins. In *Y. enterocolitica*, the conduit has been shown to be composed of a single polymerized protein, YscF [15]. Polymerization of YscF is thought to be sufficient to puncture mammalian host cell membranes. Because of the presence of homologous YscF proteins in *Yersinia* species and other bacteria that utilize TTSS [16], we hypothesized that the YscF protein itself may possess the qualities of a protective antigen. In the current study, we demonstrate for the first time that a recombinant His6-YscF protein behaves as the polymerized, native YscF purified from *Y. enterocolitica*. In addition, immunization with a purified recombinant *Y. pestis* YscF protein offers significant protection against lethal subcutaneous challenge with *Y. pestis*.

Materials and methods

Cloning

The open reading frame (ORF) of *yscF* was cloned from the pCD1 plasmid DNA of *Y. pestis* CO92 using Gateway Technology (Invitrogen, CA). Briefly, the DNA sequence corresponding to the full length *yscF* minus the stop codon was amplified by PCR with PfuTurbo (Stratagene, CA) proofreading polymerase, purified using a QuickSpin PCR purification kit (Qiagen, CA), and used for a recombination reaction with the pDONR201 entry vector (Invitrogen, CA). Plasmid DNA from positive colonies, as determined by DNA sequence analysis, was used for a second recombination with the pDEST17 vector. The final gene construct coded for a putative full length YscF protein having unnatural amino acids of MSYYHHHHHHLESTSLY KKAGF at the N-terminus and DPAFLYKVVDSRLL TKPERKLSWLLPPLSNN at the C-terminus. Both sequences are fragments from the pDEST17 vector. The C-terminal sequence results from the removal of the stop codon in YscF. Attempts to re-engineer the plasmid to remove the fusion sequences resulted in low yield bacterial expression and/or protein aggregation after purification. The final plasmid construct was verified by DNA sequencing.

Protein expression and purification

For protein expression, the plasmid DNA was transformed into the Rosetta Blue (DE3) *Escherichia coli* expression strain (Novagen, WI). Typically, 1 L of bacterial cells was grown at 37 °C in an incubator shaker for 16 h prior to harvest. Protein purification and refolding was performed in a one-step on-column refolding and purification procedure as described before [18]. Briefly, the cell paste from 2 L of cells was dissolved in 100 mL of denaturing buffer B2 (6 M GuHCl, 100 mM potassium phosphate, and 0.1% octyl glucoside, pH 8.0) and sonicated 3 × 1 min on ice. The mixture was centrifuged for 2 h at 20,000*g* at 4 °C, and the supernatant was applied to a 25 mL HisSelect HF Ni–agarose column (Sigma, MO). The immobilized protein was purified and refolded on-column by running a gradient of 100% buffer B2 to 100% buffer A2 (100 mM potassium phosphate, pH 8.0, 0.1% octyl glucoside) as described [18]. Refolded protein was eluted with 100 mM imidazole, 100 mM potassium phosphate, pH 8.0, 0.1% octyl glucoside buffer, and verified by SDS–PAGE. Positive fractions, as judged by the apparent molecular weight from SDS–PAGE gels, were dialyzed against 10 mM sodium acetate, pH 4.0, concentrated to 2–7 mg/mL in a Centriplus-3 (Millipore, MA) concentrator, aliquoted, and stored at −70 °C. The final purity was greater than 95% as judged by SDS–PAGE and LC-MS. Protein identification was confirmed by LC-ESI-MS/MS of in-gel trypsin-digested protein fragments (described later and [19]). Protein concentration was determined by UV–Vis spectroscopy from the A_{280} absorbance based on the amino acid composition. A fresh aliquot was removed from the freezer directly before each experiment.

Mass spectrometry

For protein identification by LC-ESI-MS/MS, purified protein was digested with trypsin then peptide mapping,

[1] *Abbreviations used:* His6-YscF, recombinant YscF protein; LC-ESI-MS, liquid chromatography-electrospray ionization-mass spectrometry; LD_{50}, 50% lethal dose; ORF, open reading frame; s.c., subcutaneous; TTSS, type III secretion system, *Y. enterocolitica*, *Yersinia enterocolitica*; *Y. pestis*, *Yersinia pestis*.

and de novo sequencing was performed essentially as described [19] with minor changes. Raw data were collected on an ion trap mass spectrometer, model LC-MSD Trap SL (Agilent Technology, Wilmington, DE), after pre-fractionation by reversed phase HPLC using a Zorbax SB-C8 column (1.0 × 150 mm, 3.5 mm) at a flow rate of 20 μL/min, using an enhanced scan resolution mode of 5500 (*m*/*z*)/s, with auto MS/MS analysis preference for all predicted YscF tryptic peptides being listed as doubly charged ions. The sample was injected three separate times while optimizing preferred ions settings. Raw MS/MS data were parsed against the SwissProt protein database and a custom database using Spectrum Mill MS Proteomics Workbench software (Agilent) to generate the total amino acid coverage shown in Table 2.

Circular dichroism and stability measurements

The far-UV circular dichroism spectra were recorded using a JASCO 810 (Jasco, MD) instrument at room temperature using default parameter settings. Typically, 10 spectra in the 195–250 nm range in a 1 mm path length cuvette were collected, averaged, and converted to molar ellipticity using the instrument software.

Stability measurements were performed at room temperature using the supplied titration module. Typically, the Θ_{222} signal from the instrument was recorded in the range 0–3.5 M denaturant in a 2.7 mL constant volume 1 cm path length cuvette, converted to a molar ellipticity versus denaturant concentration dataset, and plotted using the Origin 7.5 program (Microcal, MA). The stability parameters were calculated from the plots as previously described [18].

Dynamic light scattering

Measurements were performed on a DynaPro Titan instrument (Wyatt Technology, CA) using default parameter settings. Typically, the sample was filtered through a 0.2 μm syringe filter, spun for 10 min at 18,000*g* in a microcentrifuge, and the supernatant was introduced in a measuring cell maintained at 25 °C. Data were collected at default parameter settings and analyzed with the instrument's software, Dynamics. Molecular weights were calculated from the globular protein model provided by Dynamics using BSA as isotropic scattering reference.

Animal vaccinations

Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals were conducted according to the principles set forth in the *Guide for the Care and Use of Laboratory Animals* [20]. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Wild type *Y. pestis* CO92 strain was obtained from the laboratory of Dr. T. Quan, Centers for Disease Control, Fort Collins, CO [21]. For challenge studies, *Y. pestis* CO92 was initially streaked onto tryptose blood agar plates and incubated at 28 °C for 2 days. Bacterial cells were then grown for 24 h at 30 °C in heart infusion broth (HIB; Difco Laboratories, MI) supplemented with 0.2% xylose. The broth cultures were pelleted, washed with fresh HIB, adjusted to an A_{620} of 1.0 (approximately 10^9 CFU/mL), and diluted to the appropriate challenge dose.

Groups of 10 female, 8- to 10-week-old outbred (Hsd:ND4) Swiss Webster mice were inoculated with purified, recombinant YscF protein. To improve immunogenicity, each protein antigen was adsorbed with Ribi adjuvant system (RAS) R-730 monophosphoryl lipid-A (Corixa, Hamilton, MT). Two hundred microliters of each antigen–adjuvant mixture containing 20 μg of recombinant protein was administered at a single subcutaneous (s.c.) site on the backs of the animals. After 30 days, the animals were boosted with an identical dose at the same injection site. The F1–V vaccine candidate was included in the experiment to serve as a positive control.

Yersinia pestis lethal challenge

Each of the vaccinated and control animals designated to receive s.c. challenges was administered 130 50% lethal doses (LD_{50}) of wild type *Y. pestis* CO92 30 days after the booster dose. The s.c. LD_{50} for adult mice challenged with CO92 is 1.9 CFU. The mice were observed daily for 28 days, at which time the survivors were killed.

SDS–PAGE and Western blot

Samples were mixed with 4× LDS sample buffer and heated at 70 °C for 10 min before electrophoresis through NuPAGE 4–12% Bis-Tris (Invitrogen, CA) gels using Mops–SDS (Invitrogen) running buffer. Gels were stained with SimplyBlue SafeStain (Invitrogen, CA) to visualize protein. For Western blots, separated proteins were transferred to nitrocellulose membranes and probed with a 1:10,000 dilution of mouse anti-YscF serum. The secondary antibody was anti-mouse IgG antibody from a Western Breeze Chromogenic Western Blot Immunodetection kit (Invitrogen, CA) coupled to alkaline phosphatase.

Results

In the current study, we have expressed, purified, and characterized for the first time a recombinant *Y. pestis*

YscF protein. The protein was tested as a potential vaccine candidate suitable for protection against infection with *Y. pestis* in the mouse model. To assess the protein's suitability for animal studies, we first determined the recombinant protein's folding characteristics.

The recombinant His6-YscF fusion protein was expressed and purified from *E. coli* inclusion bodies (Fig. 1 and Table 1). To confirm the identity of the expressed protein, the ~15 kDa band from SDS–PAGE of the purified His6-YscF was selected for in-gel trypsin digestion and mass spectrometry (Table 2). Raw MS/MS data parsed against public and custom protein databases returned two highest scoring identifications as summarized in Table 2. Search against the public database returned "YOP proteins translocation protein F" from *Y. enterocolitica*, with three unique matching peptides and representing 34% coverage of the natural YscF protein by de novo sequencing. These fragments identically matched tryptic peptides in the *Y. pestis* YscF homolog on a BLAST search against SwissProt. Since the public database does not contain an entry for our recombinant His-tagged fusion protein, we created a custom protein database containing the predicted translation product of the gene cloned in pDEST17 expression vector. Search against the custom database returned seven unique tryptic peptide matches covering 65% of the putative amino acid sequence of our recombinant protein. These data incontrovertibly verified the purified product as the intended His6-YscF protein.

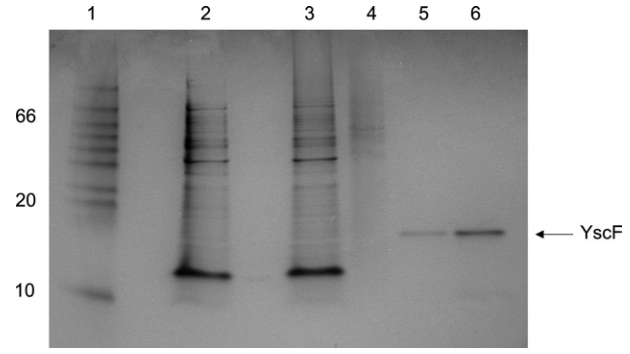

Fig. 1. Purification of YscF protein by on-column refolding. Lanes: 1, markers; 2, *E.coli* lysate; 3, cleared supernatant of denaturant-solubilized *E. coli*; 4, Ni–agarose column flow-through; 5, pooled, dialyzed fractions; and 6, concentrated YscF. To avoid protein degradation, cells were grown overnight at 30 °C without induction.

The purified protein was highly α-helical in the pH 5–7 range (Fig. 1). At pH 4.0, the spectrum changed noticeably, most likely reflecting a substantial loss of α-helical structure (Table 3). Because the protein was highly α-helical at neutral pH as expected from predictions (58% of α-helix according to methods in [17]), we are confident that the secondary structure of the refolded protein closely resembles the structure of the native protein (Fig. 2).

The properties of the recombinant protein were strongly affected by salt concentration. At low (10 mM) buffer concentration, the protein exhibited a tendency to oligomerize. At protein concentrations below 2 mg/mL, the trimer could be detected at acidic and neutral pH

Table 1
A summary of purification of recombinant YscF protein

Step	Total protein (mg)	Total yield (%)	Purity (%)
E. coli	3500[a]	100	<5
Dialyzate	15[b]	0.43	>95
Concentrated protein	12[b]	0.34	>95

[a] The number corresponds to the total weight of the harvested cells from 1 L of culture.
[b] Total protein content was determined spectrophotometrically based on calculated amino acid composition of the purified YscF protein.

Table 3
α-Helical content of recombinant YscF protein at different pH

pH	Percentage of α-helix, measured[a]
4	20
5	33
6	37
7	38

[a] Calculations were based on the molecular ellipticity per residue value at 222 nm according to formula % α-helix = Θ_{222}/3298*(−10) [22]. Samples were measured in 10 mM buffer, potassium phosphate (pH 6–7) or sodium acetate (pH 4–5).

Table 2
Protein identification by LC-ESI-MS/MS

Protein database searched[a]	Protein name (accession)	Coverage[b]	Observed amino acid sequences[c]			
SwissProt	YEREN[d] (Q01247)	34%	MSNFSGFTKG	NDIADLDAVA	QTLKKPADDA	NKAVNDSIAA
			LKDTPDNPAL	LADLQHSINK	WSVIYNISST	IVRSMKDLMQ
			GILQKFP			
Custom	His_6-YscF	65%	MSYYHHHHHH	LESTSLYKKA	GFMSNFSGFT	KGTDIADLDA
			VAQTLKKPAD	DANKAVNDSI	AALKDKPDNP	ALLADLQHSI
			NKWSVIYNIN	STIVRSMKDL	MQGILQKFPD	PAFLYKVVDS
			RLLTKPERKL	SWLLPPLSNN		

[a] Protein database used to parse MS/MS raw data. Custom database contained the complete putative amino acid sequence predicted from the cloned *his6-yscF* gene. Databases were analyzed using SpectrumMill software as described in the text.
[b] Amino acid coverage of the protein with the highest scoring hit is shown as observed AA/total AA in percentage.
[c] Total amino acid sequence of the protein having the highest scoring hit is shown in increments of 10 AA. Amino acids experimentally observed by de novo sequencing are underlined.
[d] The YscF homolog from *Y. enterocolitica* scored highest in searching the published protein database because the His_6-YscF entry is not present.

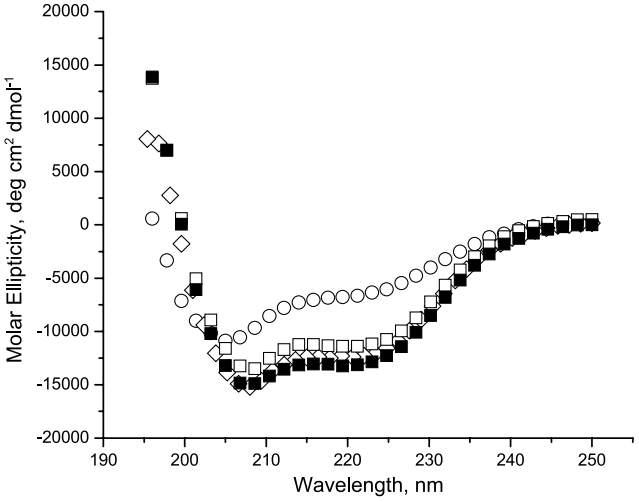

Fig. 2. Recombinant YscF protein is a α-helical protein under neutral conditions. Far-UV CD spectra of recombinant YscF protein at pH 7.0 (■), 6.0 (◇), 5.0 (□), and 4.0 (○). Spectra were recorded at room temperature at 0.2 mg/mL protein concentration in 10 mM buffers: pH 4–5, sodium acetate, and pH 6–7, potassium phosphate.

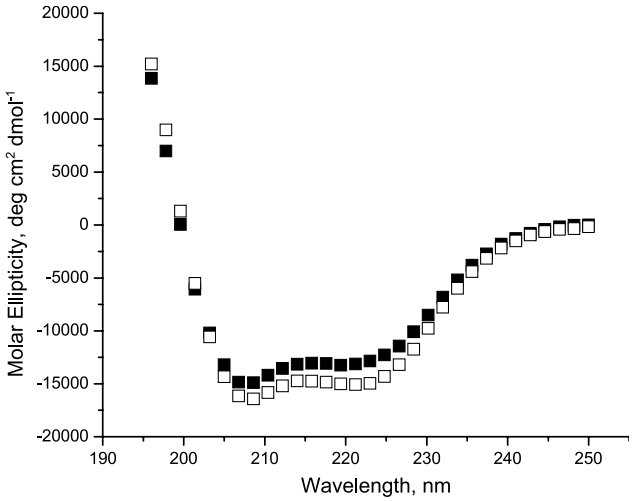

Fig. 3. YscF remains α-helical under neutral pH conditions in the presence of salt despite aggregation. CD spectra of YscF were recorded in 10 mM potassium phosphate, pH 7.0, in the presence (■) and absence (□) of 150 mM NaCl. Data in the absence of salt were included for comparison.

conditions (Table 4 and discussed later). At protein concentrations above 7 mg/mL, the population of higher-order (more than three) aggregates prevented size distribution measurements by dynamic light measurements. The aggregation was even more pronounced when experiments were performed at physiological salt concentrations (data not shown), but the spectrum remained α-helical and very similar to the one recorded in the absence of salt (Fig. 3). Our data suggest that the protein may form higher-order oligomers with minimal changes in the secondary structure. These observations appear to be consistent with notion that YscF protein has the capacity to polymerize into needle-like structures under physiological conditions.

To determine if salt concentration affected the stability of the protein, we performed denaturant unfolding in the presence and absence of salt. At neutral pH conditions, the protein was highly flexible at low (10 mM) buffer concentrations, but the transition region was relatively well defined at physiological (>100 mM) salt concentrations (Fig. 4) and overall protein stability was measurable (Table 5). At acidic conditions, however, the

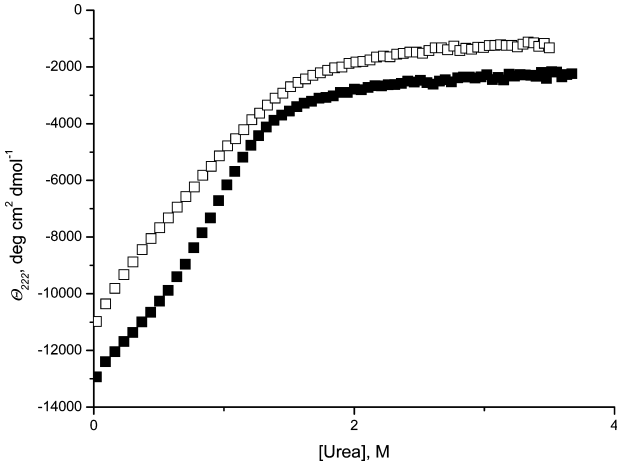

Fig. 4. Recombinant YscF protein is relatively stable only under neutral conditions in the presence of physiological concentrations of salt. Representative CD-monitored urea unfolding curves of YscF in the presence (■) and absence (□) of 150 mM salt in 10 mM potassium phosphate, pH 7.0. The data at pH 4.0 were almost identical to the data recorded in the absence of salt and were not included for clarity.

Table 4
Oligomerization of YscF protein detected by dynamic light scattering

pH	Molecular weight[a] (kDa)	Oligomerization state
4.0	43.5	Trimer
7.0	40.0[b]	Trimer

[a] The calculations were based on a globular model of proteins as provided with the instrument's software. Measurements were performed in 10 mM potassium phosphate (pH 7.0) or 10 mM sodium acetate (pH 4.0) at 1.68 mg/mL protein concentration. Calculated MW of YscF is 15.8 kDa.

[b] The data analysis suggested a small (<0.1% of mass) amount of aggregate with a MW of 5.5 MDa.

Table 5
Thermodynamic stability of recombinant YscF protein

pH	[NaCl] (mM)	ΔG^0 (kJ mol^{-1})	C_m (M)	m (kJ M^{-1} mol^{-1})
7.0	0	ND[a]	ND[a]	ND[a]
	100	11.4 ± 0.6	1.17 ± 0.02	9.7 ± 0.3
	150	10.2 ± 0.6	1.02 ± 0.02	9.9 ± 0.3
4.0	0	ND[a]	ND[a]	ND[a]
	150	ND[a]	ND[a]	ND[a]

All measurements were made in 10 mM potassium phosphate (pH 7.0) or 10 mM sodium acetate (pH 4.0). Parameters were determined for unfolding in urea as described in Materials and methods.

[a] ND, Not determined due to lack of measurable transition region.

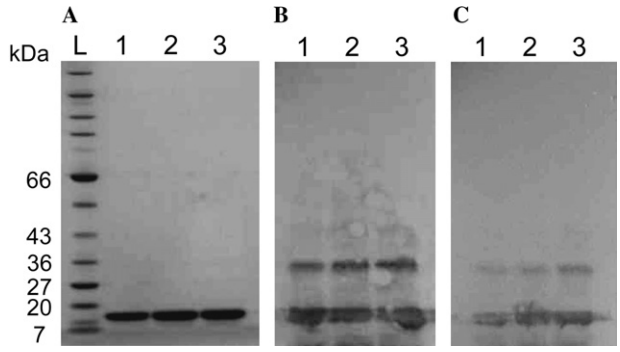

Fig. 5. Immunization with recombinant YscF yields YscF-specific antibodies. Sera from immunized mice were used as primary antibodies in detection of YscF protein. (A) Coomassie-stained gel of purified YscF protein. (B, C) Western blots of purified YscF protein at 1:1000 and 1:10,000 dilutions of the mice sera, respectively. Lanes: L, molecular weight markers; 1, 2 μg; 2, 4 μg; and 3, 6 μg of protein loaded per lane. The bands at approximately 36 and 43 kDa MW correspond to oligomers of the YscF protein. Control Western blots with proteins other than YscF were negative (data not shown).

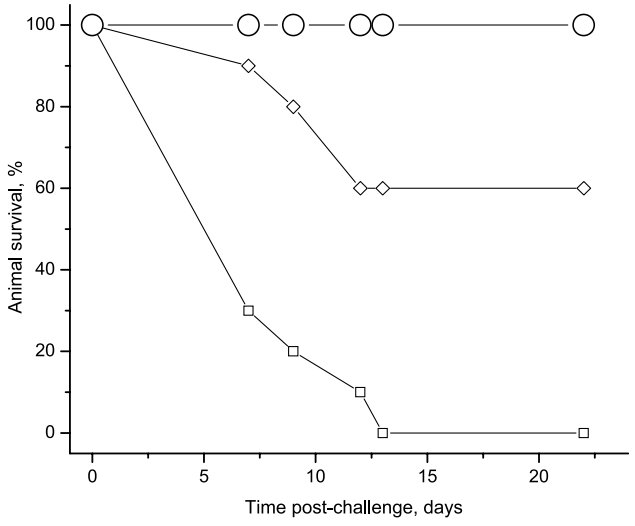

Fig. 6. Immunization with recombinant YscF protein confers significant protection against challenge with live wild type *Yersinia pestis* CO92 strain. The animals were immunized with recombinant YscF protein (◇), recombinant F1–V fusion protein (○) or adjuvant only (□) and challenged with wild type *Y. pestis* CO92.

protein did not show a tertiary structure regardless of the salt concentration (data not shown). We concluded that the protein's tertiary structure is relatively stable only under neutral pH conditions in the presence of physiological salt concentrations.

Characterization of the purified YscF indicates that the recombinant protein is highly α-helical and can assemble itself into higher-order oligomers under physiological conditions. The tertiary structure of the protein was relatively stable under neutral conditions but practically non-existent under acidic conditions. Since our data are in agreement with experimental observations on purified, polymerized native YscF from *Y. enterocolitica* by Hoiczyk and Bloebel [15], we postulate that the recombinant protein is properly folded and resembles the native YscF protein.

Purified YscF protein was formulated with R-730 emulsion and used to inoculate outbred mice prior to s.c. challenge with 130 LD_{50} *Y. pestis*. After the second vaccination, the animals exhibited a high level of YscF-specific serum antibodies as indicated by immunoblot (Fig. 5). Six out of ten YscF vaccinated mice survived a lethal challenge with *Y. pestis* while 100% of the mice that were inoculated with R-730 adjuvant alone succumbed to infection (Fig. 6). These results support the hypothesis that YscF immunization can provide significant protection against wild type *Y. pestis* as an individual antigen ($P = 0.05$). As expected, vaccination with the F1–V fusion protein provided 100% protection against the wild type pathogen ($P = 0.01$).

Discussion

The *Y. pestis* bacterium is a human pathogen for which an effective prophylactic is currently not available. Normally extracellular, the bacterium is easily internalized by macrophages and able to survive inside the cell while being distributed throughout the host's body. While outside the cell, the bacterium is normally encapsulated by the F1 protein and the F1 antigen is an effective immune target [3]. Upon bacterial attachment and internalization, the normal cellular signaling network of the host is effectively silenced by secreted Yop effectors offering the pathogen effective protection from the host's immune surveillance.

The Yop delivery is mediated by the YscF protein [15] and the YscF peptide itself is a logical choice for evaluation as a potential protective antigen. YscF-induced protection could potentially arise because of enhanced immune recognition of surface-exposed YscF protein. Alternatively, recognition of surface-exposed YscF by anti-YscF antibodies may play a role in the prevention of virulence factor delivery. Here, we demonstrate for the first time that the recombinant YscF protein resembled the native species and immunization with the recombinant protein has the capacity to offer significant protection against lethal plague challenge with wild type *Y. pestis* in a subcutaneous infection model.

A previously described F1–V fusion protein employed as historical control in this study is a highly effective recombinant fusion protein vaccine against s.c. plague infection (Fig. 6) and numerous previous studies [2,23]. However, the nature of the two-component fusion protein could allow for the development *Y. pestis* strains with the capacity to evade F1–V vaccine induced immunity. Complex vaccines made of many constituents, including F1–V based derivatives, may provide synergistic prophylaxis and prove to be more difficult to evade.

For this purpose, it is necessary to elucidate additional *Y. pestis* antigens with the capacity to provide a protective immune response against plague. The primary goal of this study was to test YscF as a possible alternative or adjunct protective vaccine antigen against bubonic plague. Vaccination of mice with the recombinant protein provided significant protection against subcutaneous challenge with wild type *Y. pestis* CO92 bacterium. In comparison, this vaccine antigen represented 60% of the efficacy provided by the F1–V protein standard. To better understand these survival results, we also tested whether the recombinant YscF protein was properly folded and structurally similar to the native protein. Results presented here show that the purified His6-YscF protein folded into a form appeared to be in a relatively stable conformation and also resembled secondary structure as predicted by computational models. Thus, we believe that the biological data are not biased by improper folding and are likely to be representative of an effect by the natural YscF protein. Therefore, YscF protein has the potential to provide added protection against subcutaneous plague infection and YscF may contribute to the development of enhanced vaccines for plague.

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